Uptake, transport and accumulation of nicotine by the Golden Potho (Epipremnum aureum): the central role of root pressure

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Summary

The roots of Epipremnum aureum, though not synthesizing nicotine themselves, take up exogenously fed nicotine as a xenobiotic. The alkaloid is subsequently translocated to the leaves, via the xylem path, where it accumulates in the mesophyll up to levels comparable with nicotine-rich Nicotiana species. The Epipremnum plants accept nicotine only up to a distinct level; saturation is reached after about 10 days. All mature, non-senescent leaves accumulate the same amount of nicotine. By different experimental approaches, unequivocal evidence could be provided that root pressure is the 'translocative force' for nicotine transport in E. aureum. Xylem sap exudates, collected from shoot stumps that were connected to an intact root system immersed in nicotine solution were analyzed for nicotine content. Nicotine uptake from the medium by the root and its subsequent transfer into the xylem of the shoot persisted for more than 10 h without measurable decline of the transport rate, provided the nicotine concentrations applied were <0.05%. In intact plants, where both components of water transport in the xylem — root pressure and transpirative water flow — are in operation, no surplus transport of nicotine from the roots into the leaves took place beyond the level observed in amputated plants. Under the influence of inhibitors of root respiration, nicotine uptake was halted slowly in case of oxygen deprivation and in case of cyanide, or it stopped very rapidly when CCCP, an uncoupler of mitochondrial ATP formation, was applied to the roots. This threshold of toxicity against the xenobiotic was established by dose effect curves for nicotine sensitivity of the roots for root respiration and by transpiration measurements. Leaves, bearing a heavy 'nicotine load', showed symptoms of senescence only after 3–6 weeks, as indicated by a decline in the chlorophyll content, the chl a/b ratio, and the maximal quantum yield efficiency (Fv/Fm), and by an increase in catalase activity. Our results

KEYWORDS

Epipremnum aureum; Golden potho; Nicotine uptake; Xenobiotics; Ion trap; Root pressure; Leaf senescence

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provide insight into the mechanisms of uptake, transport and storage of nicotine as a xenobiotic.

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Introduction

Originally, we observed that $^{14}$C nicotine fed to the roots of *Epipremnum aureum* was taken up easily by this ornamental plant, known as ‘Golden Potho’. Although *E. aureum* does not synthesize nicotine itself, considerable amounts of nicotine were deposited in the leaves (Schmitz, 1995). Therefore, nicotine has to be considered a xenobiotic substance (Sandermann, 1992; Martinoia et al., 2000) with respect to *E. aureum*. In contrast, *Ficus benjamina*—the ‘weeping fig’—, another favorite plant for indoor greening purposes, does not at all take up nicotine from a root medium (Schmitz, 1995). Hence, we became interested in this seemingly uncommon capability of *E. aureum*, which—to our knowledge—has not been reported before. Consequently, the present investigation was initiated. Our main focus is directed on nicotine uptake and transport mechanisms and on nicotine storage in the leaves of *E. aureum*. A major experimental tool was xylem sap analysis. Since this species exhibits pronounced and long-lasting root pressure activity, it was possible to collect xylem sap exudate for many hours. In this context, the effects of inhibition of root pressure via inhibitor-induced ATP deprivation of the root were also studied. Furthermore, we were interested in the (stress) physiological consequences of nicotine accumulation in the leaves with respect to induced leaf senescence.

Materials and methods

Plant material

All experiments were carried out with the Golden Potho (devils ivy) *E. aureum* Lind and Andre (=*Epipremnum pinnatum* L.). Hydroponic plants of *E. aureum* were obtained from Geselka GmbH, Köln, FRG. The mother plants were cloned, that is, they were fractioned into one-leaf stem cuttings that were grown on Vermiculite for 10–12 weeks in the greenhouse at $\pm 25^\circ C$ under natural light and, supplementary, with artificial white light (Phillips SON-T-AGRO 400; photon flux density (PFD) approximately $500 \mu \text{mol m}^{-2} \text{s}^{-1}$). Commercial fertilizer (‘Wuxal Super’; N/P/K 8:8:6 plus micronutrients) was administered weekly to the plants. Experiments were mostly carried out with well-rooted plants that had developed 4–6 new leaves after vegetative propagation (cloning).

General growth and incubation conditions

For the experiments, the plants were transferred into 100-ml Erlenmeyer-flasks or 250-ml beakers containing 25–100 ml incubation medium, depending on the experiment; the roots were kept submersed. The opening of the flasks/beakers was covered with aluminum foil or Parafilm wrapped around the stem in order to prevent evaporation. The incubation medium consisted of Knop’s nutrient solution (Hewitt, 1966); 0.03% chloroamphenicol was included to suppress the growth of nicotine-consuming bacteria (Eberhardt, 1995). Alternatively, Hoagland No. 3 nutrient solution, supplemented with Arnon’s A-4 micronutrient solution (Hoagland and Arnon, 1950), was used. The osmolarity of the incubation media was $0.05 \pm 0.02 \text{ osmol kg}^{-1}$.

The dissociation equilibrium between the electroneutral (nic) and the cationic form of nicotine (nic$^+$) depends on the pH of the solution (The Merck Index, 1976). At pH 7, about 50% of the nicotine is in the nic-form (electroneutral), calculated on the basis of an apparent pK$\text{a}_a$-value of 8.02 for nicotine, whereas at pH 5.3 (the pH-value of *E. aureum* root homogenates) only about 1% is in the apolar/lipophilic form according to the equation:

$$\text{pH} - 8.02 = \log \frac{[\text{nic}^+]}{[\text{nic}]}.$$  \hspace{1cm} (1)

The pH-value of the incubation medium either adjusted to pH 7, which is the normal pH-value for nutrient solutions, or pH 5.3, which is the pH-value of *E. aureum* root homogenates.

Pulse-chase incubation of intact plants with $^{14}$C nicotine

Two days before the pulse-chase experiment was started, the *E. aureum* plants were removed from the greenhouse and preadapted to the conditions of the growth chamber, where the nicotine incubation (pulse) and the subsequent chase period were to be administered. The settings were as follows: 25 $^\circ C$, 70% relative humidity, continuous light (fluorescent tubes ‘cool white’), PFD was approximately...
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150 $\mu$mol m$^{-2}$s$^{-1}$. A 48 h nicotine pulse was administered to the roots by adding (pyrrolidone-2-$^{14}$C)-DL-nicotine to Knop’s medium (pH 7) to yield a final concentration of 0.05% (v/v) on nicotine; the specific activity was 2.4 MBq mmol$^{-1}$ (7.4 kBq ml$^{-1}$). During incubation, the flasks were agitated slowly on a shaker. At the end of the pulse period the plants were either analyzed directly for nicotine or were replanted into Vermiculite and watered with nicotine-free Knop’s medium for the chase periods, applied. The harvested plant material was wrapped in punctured aluminum foil, deep-frozen with liquid nitrogen (LN$_2$), and stored at $-20$ °C until the analyses were carried out. Xylem sap aliquots were shock-frozen (LN$_2$/$-20$ °C) in 1.5 ml tubes.

The in vivo measuring of incorporated label of whole leaves was done with a large-window $^{14}$C xenon monitor (Contamat FHT 111 M; FAG Schweinfurt, FRG). The counter was positioned 2 mm above the leaf surface. For one recording, three consecutive 2 min-measurements (Bq cm$^{-2}$) of a certain leaf were taken and averaged. The standard errors were in the range of 2%. Due to the relatively low $^{14}$C counting sensitivity inherent to this detection method, the values do not represent the absolute of incorporated labeled nicotine. However, this fact does not distort the time curve of labeling and the final distribution of incorporated $^{14}$C. An autoradiogram from a whole plant was obtained after a 2 day pulse, followed by a 10 day chase period. The plant was tightly pressed against Hyperfilm (Amersham) for 2 weeks at $-20$ °C.

**Incubation of excised roots with unlabeled nicotine and collection of xylem sap**

The shoots of *E. aureum* plants were cut off about 5 cm above the root base. The adhering Vermiculite was carefully rinsed-off the roots of the amputated plants, which were then placed in 250 ml-beakers positioned in a 25 °C water bath. The beakers contained 50 (100) ml of Hoagland’s medium adjusted to pH 5.3. The roots were aerated by aid of an aquarium air pump. A 1 ml-pipette tip (blue tip) was put upside-down on top of the cut surface of each root explant and sealed from the outside with vaseline in order to make the setup water tight. The xylem sap exudate was collected at regular time intervals with a 100 $\mu$l syringe which was inserted through the narrow outlet of the tip. The xylem sap flow ($\mu$l h$^{-1}$) reached a constant rate after about 2 h. At that stage of the experiment, the nicotine-free Hoagland medium was replaced by fresh nutrient solution, which additionally contained 0.05% (v/v) unlabeled nicotine. In order to maintain a non-obstructed xylem sap exudation for an extended period of time, a 2 mm slice was cut off the stump surface after every second xylem-sap ‘harvest’.

**Nicotine incubation of intact plants in the presence of inhibitors**

*E. aureum* plants were transferred from the Vermiculite into beakers that contained Hoagland’s nutrient medium adjusted to pH 7. At this pH-value, the concentration of nic equals the concentration of nic+ (see above). After preadaptation to growth chamber conditions, 0.05% unlabeled nicotine was added. In addition, either KCN or CCCP (carbonyl cyanide-m-chlorophenylhydrazone) was included to yield a final concentration of 10$^{-3}$ M (cyanide) or 10$^{-5}$ M (CCCP), respectively. In the third inhibition treatment, nitrogen instead of air was bubbled through the incubation medium in order to establish anoxic conditions. In case of the N$_2$ treatment, a pinhole in the Parafilm cover enabled the ventilated gas to escape. The transpired water volume was determined by differential weighing of the whole setup (plants and beakers). Six plants plus six inhibitor-free controls were used for each inhibition variant.

After 24 h of incubation, all shoots were cut off about 5 cm above the root base and dissected. The root bodies including the shoot stumps remained in the incubation medium for another 2 h to collect xylem sap. This could be done only with the controls because at that stage of the experiment the inhibitor-treated plants no longer exuded xylem sap. Nevertheless, a small volume of xylem sap could still be obtained by cautiously squeezing the stem stumps.

The leaf laminae were separated from the shoots, cut into pieces and frozen (LN$_2$/$-20$ °C), the stems were discarded. For nicotine analysis, batch samples of leaf tissue were prepared by combining equal fresh weight aliquots of the leaves from each of three plants. This makes up two mixed samples per inhibitor treatment and two minus-nicotine samples.

**Analysis of nicotine content**

Two different nicotine assays were employed for tissue samples and for xylem sap aliquots, respectively. The nicotine contents of gram amounts of leaf- and root material were analyzed after DIN-instruction No. 10241 (1982), an established standard procedure for quantitative nicotine analysis of
tobacco samples. Nicotine was extracted with 8 N NaOH from tissue homogenates and dissolved in saturated NaCl solution. It was then separated from the slurry by water-vapor distillation and captured in 1 N H₂SO₄. The distillate was brought to a distinct volume, and nicotine was measured from aliquots spectrophotometrically at 254 nm. Recovery for nicotine standards was 98.3 ± 5.2%.

The nicotine content of small tissue samples (10–40 mg fresh weight) and of xylem sap aliquots was determined by reversed-phase HPLC. Plant tissue samples were pulverized with LN₂. The frozen powder was extracted for 20 min at room temperature with a methanol/acetic acid mixture (v/v, 3:5). The extract was clarified by centrifugation (40,000 g for 10 min). The Merck EXTRELUT® system was used to separate the nicotine from the leaf extract (Kohl et al., 1983; McCaskill et al., 1988). Three-ml-aliquots of the extract were loaded on an EXTRELUT column. After 15 min, the column was alkalized by an air/NH₃ stream for 10 min. Uncharged nicotine (nic) was eluted with 18 ml CHCl₃. The eluate was brought to dryness by vacuum distillation of the solvent. The residue was redissolved in a small volume of methanol and pressed through a syringe, fitted with a 40 µm filter disk. Aliquots of the filtrate were subjected to isocratic HPLC, employing an RP column (Bakerbond® Wide-Pore C8; RP-7105-1; particle size 5 µm; 4.6 × 100 mm²).

Leaf samples were eluted with a mixture of methanol/H₂O/triethanolamine (v/v/v, 120:180:225), the flow rate was 1.2 ml min⁻¹. Xylem sap aliquots were chromatographed the same way. In this case however, the elution was carried out with a 1:1 mixture of methanol and 0.2% ortho-phosphoric acid at pH 7.25, adjusted with TEA (Verpoorte and Baerheim-Svendsen, 1984).

Chlorophyll fluorescence, chlorophyll content, and chlorophyll a/b-ratio

For determining the F₅₀/F₅₉ ratio as a measure for the maximum quantum efficiency (Schreiber et al., 1986) of E. aureum leaves, the Mini-PAM field-fluorimeter (Walz, Effeltrich, Germany) was used. Green sections of the variegated leaves were predarkened for 20 min (for complete reduction of the photosystem II reaction centers) with special shutter clamps. Consecutive measurements were always taken at the same (marked) leaf positions. F₀ was measured with a red light impulse (650 nm 5 s; PFD: 0.30 μmol m⁻² s⁻¹), F₅₉ was determined with a 0.8 s white light pulse (PFD was approximately 9000 μmol m⁻² s⁻¹).

Total chlorophyll content and the chl a/b-ratio of the leaves was analyzed after Hiscox and Israelstam (1979). The leaf material (0.1 g fresh weight) was extracted with 10 ml DMSO for 17 h at 65 °C. The chlorophyll concentrations were then calculated from the O.D-values at 663 and 645 nm according to Arnon (1949).

Root respiration

One-leaf cuttings from E. aureum were cultivated in Vermiculite® in a humid chamber at 25 °C until (after about 2 week) an unbranched adventitious root of 8–10 cm length had developed at the node. Respiration rates (μmol O₂ g⁻¹ root fresh weight) were measured at the beginning and at the end of a 24 h period of nicotine incubation. During incubation, the plants were irradiated (PFD: 100 μmol quanta m⁻² s⁻¹) of 0.05% on nicotine. The incubation tubes contained one plant and Knop’s medium (pH 5.3) made of 0.05% on nicotine. The tubes were aerated and kept at 22.5 °C. Polarography was carried out in a self-made 8 cm high stirred cuvette, fitted at the bottom with a disk-type Clark electrode (Hansatech DW-1, Bachofen, Reutlingen,) cuvette. The cuvette contained 5 ml Knop’s medium (omitting Fe²⁺). The root was inserted into the cuvette from the top; the root base and the adhering shoot remained outside the chamber. The cuvette was carefully sealed against the air with plasticine without injuring the root base.

Results

Nicotine accumulation in the leaves

In the first pulse-chase experiment, 0.05% ¹⁴C-labeled nicotine was administered to the roots of 3-month-old, well-rooted E. aureum cuttings during a 48-h pulse followed by a 30-day chase period. Figure 1 shows that after 3-day chase, more than half of the nicotine taken up by the plant (Σ = 100%) was still located in the root, whereas after 30 days, 97% of the nicotine captured by the plant, had been translocated into the leaves. An identical experiment was carried out with F. benjamina (weeping fig) with a completely different outcome: even after 1 month, nicotine could only be traced in the roots, and the shoots were absolutely nicotine-free (Schmitz, 1995). This result prompted us to consider this ability of E. aureum to be a special feature of this species worthy of studying in detail.
In a second experiment, again a 48 h-pulse with \(^{14}\text{C}\) nicotine was applied to \(E. \text{ aureum}\) plants. An in situ autoradiogram of a complete plant was taken after 10 days of chase (Fig. 2). Obviously, the distribution of nicotine across the leaf lamina was rather inhomogeneous. \(^{14}\text{C}\) label turned out to be predominantly located in the peripheral, that is, the younger parts of the leaves, whereas the central part of the laminae as well as the leaf bases, the petioles, and the shoot were practically free of label. Regarding the fact that transpiration is most intense in the young, peripheral part of dicot leaves (Peterson and Edgington, 1975). This coincidence between transpiration intensity and nicotine labeling indicates that nicotine is translocated from the roots to the leaves of \(E. \text{ aureum}\) by the xylem path.

For one single plant bearing six leaves, the time course for accumulation of \(^{14}\text{C}\) nicotine within the shoot was recorded with a large-window \(^{14}\text{C}\) counter. Saturation-type curves were obtained for all leaves (Fig. 3). Evidently, leaves of \(E. \text{ aureum}\) incorporated only a limited amount of nicotine — independent of leaf size and leaf position: All mature leaves (No. 2–5) stored approximately the same amount of nicotine. Only the oldest, possibly senescent leaf (No. 1) and also the youngest leaf (No. 6) accumulated much smaller amounts of nicotine. In all leaves, nicotine-uptake leveled off after about 10 days. Thereafter, \(^{14}\text{C}\) labeling of the leaves remained constant up to 30 days of chase. Thus, no relocation of nicotine or of nicotine-derived labeled metabolites took place.

Quantitative information about the time course of nicotine accumulation in \(E. \text{ aureum}\) leaves was obtained from two large plants (4–10 leaves), which were nicotine treated as described above, except that the roots were immersed in 0.1% nicotine. Thereafter, the plants were replanted in Vermiculite\(^{\text{15}}\) for 35 days of chase. The nicotine content was regularly determined for 5 weeks. The same uptake curve as in the previous experiment evolved (Fig. 4). The final level of the nicotine concentration, reached after 2 weeks, was 3–4 mg g\(^{-1}\) dry weight. These plants were also used for a senescence study (see below).

**Influence of nicotine on root respiration and on transpiration**

It was essential to know which nicotine concentration could be safely applied to the roots of \(E. \text{ aureum}\) without causing damage. Only then could...
more specific uptake experiments be designed. One parameter thought to yield information about the nicotine susceptibility of roots was transpiration. Transpiration rates were measured by differential weighing. It turned out that nicotine concentrations \( > 0.05\% \) caused a 80\% decrease of the transpiration rate within 2 days, whereas at \( \leq 0.05\% \) nicotine water uptake was not disturbed and hence the transpiration rate remained unaffected (Fig. 5).

Another parameter for testing nicotine-induced root impairment was respiration. A slow but increasing drop of the in vivo root respiration rate was observed with rising nicotine concentrations (Fig. 6). Yet, the inhibition of \( \text{O}_2\) consumption was negligible at 0.05\% nicotine. Rather unexpectedly, the control plants (receiving no nicotine) exhibited a 40\% increase in respiration after 24 h as compared to the initial \( \text{O}_2\) consumption rate, which was 170 \( \mu\text{g} \text{O}_2\text{g}^{-1}\text{min}^{-1} \) (---). For that reason, the whole curve shows a shift to higher values. For that reason, the respiration rate in the presence of 0.05\% nicotine was not lower than the initial rate of oxygen consumption. This in turn means that oxidative ATP formation in the root—as a prerequisite for root pressure (see below)—is not hampered by nicotine under these conditions. One may suggest that this ‘enhancement effect’ is possibly caused by improved oxygen and nutrient availability during the 24 h of nicotine incubation as compared to plants that had been removed from the Vermiculite\(^{6}\) substrate just before the experiment began. In agreement with the transpiration measurements, these results indicate that 0.05\% nicotine can be safely used for nicotine uptake experiments with \textit{E. aureum}, provided the \( \text{O}_2\) and nutrient supply of the roots is optimal. In the following experiment, care was taken to ensure this.

**Long-distance transport of nicotine**

Principally, xenobiotics can either be translocated in the phloem (\textit{Hsu and Kleier, 1996}) or in the xylem (\textit{Jakob and Neumann, 1987}). In the genus \textit{Nicoti-
ana, the transport route for nicotine from the roots (as the site of synthesis) to the leaves (as the site of accumulation) is the xylem (Tso, 1972). Does this hold true for E. aureum and nicotine as a xenobiotic compound, too? Indirect evidence has already been presented that nicotine moves along with the xylem water flux in E. aureum stems (Fig. 2). Additional evidence for nicotine being a xylem-mobile compound in E. aureum, will be presented below.

Decapitated E. aureum plants continue to exude xylem sap from the shoot stump for hours. The exudate was collected from the stump for 30 h at regular time intervals, and all samples were analyzed separately for nicotine. The nicotine concentration of the xylem sap approached the concentration of the external 0.05% nicotine solution within about 10 h and remained constant thereafter for \( \geq 20 \) h (Fig. 7). In addition, for any given sampling time, the total amount of nicotine that had passed through the xylem was calculated from the summed-up nicotine contents and the respective volumes of xylem sap. The values, obtained by this way from plant No. 3, were plotted against time of ‘harvest’. The curve for the time-integrated total amount of nicotine collected from the xylem exudate is shown in Fig. 8 (—). The dashed line included shows the hypothetical curve for the increase in total nicotine, calculated on the basis of the initial rate of exudation according to the equation:

\[
\text{nic} = \int_{t_i}^{t} c_{\text{nic}} V \, dt, \tag{2}
\]

where \( c \) is the nicotine concentration and \( V \) the exudate volume.

From about 20 h on, this theoretical curve diverged from the (lower) experimental curve. After about 40 h, nicotine transport finally ceased completely. Since the nicotine concentration in the xylem sap was constantly between 10 and 40 h, as indicated by the data of Fig. 7, it must have been the rate of xylem sap exudation (\( \mu l \text{h}^{-1} \)) that started to slow down at \( \geq 20 \) h. In other words, in the presence of 0.05% nicotine, root pressure remained unaffected for just about 20 h—and so did nicotine transport! This leads to the conclusion that the mechanisms responsible for root pressure (Kramer and Boyer, 1995) are directly engaged in nicotine transport as well.

Comparison of nicotine uptake into intact and decapitated plants

So far, we have shown that root pressure is in fact involved in nicotine transport. However, the contribution of transpirative water flow to nicotine transport still remains unclear. The question is: is there a preference in E. aureum for the one or the other of the two water-driving forces? A comparison with respect to their relative contributions to nicotine transport was done by evaluating the nicotine transport in intact and in decapitated plants (roots plus shoot stump). The roots of the E. aureum plants were incubated with unlabeled 0.05% nicotine for 48 h as described above.
In the case of the intact plants, the pre- and post-weight of the complete experimental setup (plants plus incubation flask plus incubation medium) were determined and the total amount of water that had been transpired during 48 h was calculated as Δ weight. At the end of the incubation period, the stems of the intact plants were cut off and analyzed for nicotine content. The decapitated plants were handled as in the preceding experiment. The summed-up nicotine content of the xylem exudates, collected from decapitated plants within 48 h of incubation yielded the amount of nicotine that was translocated by root pressure alone.

The following picture evolves (Table 1; columns 1 and 2): Just about the same total amount of nicotine was taken up by the roots of non-decapitated and of decapitated plants. In contrast, a large difference between the two groups of plants exists with regard to ‘total water flow’ (column 3). Transpiration exceeded root pressure by a factor of 4–5. In the view of these results, one would expect that the amount of translocated nicotine is 4–5 times higher than in decapitated plants.

Instead, the nicotine concentrations in the xylem exudates were 3–4 times higher than the calculated nicotine concentration in the xylem sap of intact E. aureum plants (column 5), which was calculated by relating the data for the transpired water volume (column 4) to the data on total content of nicotine in the shoots (column 1). It turned out that approximately the same amount of nicotine was translocated in the presence and in the absence of the transpirative water flow. This forces the conclusion that nicotine transport depends exclusively on root pressure and the superimposed transpirative water flow, though much more intense, only ‘dilutes’ the nicotine load in the xylem.

Inhibition of root pressure and nicotine transport

The finding that root pressure is the one and only ‘driving force’ for transport of nicotine in E. aureum was confirmed by an inhibitor experiment where pressure was annihilated by oxygen deprivation (by ventilating the roots with N2) and by inhibition of oxidative phosphorylation by KCN and CCCP, respectively. Table 2 summarizes the results. Under hypoxic conditions (plants 3 and 4), the xylem sap did not contain any nicotine after 24 h of exposure, whereas the exudates of the control plants (plants 1 and 2) were enriched in nicotine. Surprisingly, however, the nicotine content of the leaves of N2-treated plants was not at all lower than that of the controls. In accordance, the root concentration factor (RCF) after Shone et al. (1974) was just as high under anoxic conditions as it was in the controls. For explanation: an RCF-value of 2.7 means that the concentration of nicotine within the root exceeds the concentration in the incubation medium (0.5 mg ml\(^{-1}\)) by a factor of 2.7 (1.35 mg g\(^{-1}\) fresh weight). Referring to plants 5 and 6, the same results were obtained with KCN, an inhibitor of cytochrome oxidase. The outcome of the inhibitor experiment was different, however, when the protonophor and uncoupler CCCP (Felle and Bentrup, 1977) were employed. Under this inhibition regime, the leaves did not contain any nicotine and only a small amount of nicotine was found in the roots. Hence, the RCF-value was only about 0.75. Expectantly, the xylem sap did not contain any nicotine. Taken together, these results suggest that complete inhibition of nicotine uptake and transport must have occurred much more rapidly in the case of CCCP than in that of N2-

Table 1. Nicotine uptake and transport in intact and decapitated E. aureum plants

<table>
<thead>
<tr>
<th>Plant number</th>
<th>Nicotine uptake per plant (mg)</th>
<th>Nicotine uptake per root dry weight (mg g(^{-1}))</th>
<th>Transported water volume per root dry weight (ml g(^{-1}))</th>
<th>Nicotine transport per water volume (mg ml(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intact plants (water flow includes transpiration and root pressure)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>0.91</td>
<td>8.6</td>
<td>70.1</td>
<td>0.121</td>
</tr>
<tr>
<td>2</td>
<td>3.25</td>
<td>13.5</td>
<td>66.4</td>
<td>0.203</td>
</tr>
<tr>
<td>3</td>
<td>3.12</td>
<td>11.1</td>
<td>51.4</td>
<td>0.215</td>
</tr>
<tr>
<td>Decapitated plants (water flow due to root pressure only)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>2.3</td>
<td>3.7</td>
<td>8.6</td>
<td>0.426</td>
</tr>
<tr>
<td>5</td>
<td>11.0</td>
<td>10.0</td>
<td>17.4</td>
<td>0.573</td>
</tr>
<tr>
<td>6</td>
<td>5.5</td>
<td>7.7</td>
<td>14.2</td>
<td>0.513</td>
</tr>
</tbody>
</table>

The roots were exposed to 0.05% nicotine for 48 h. In intact plants, the total water loss based on transpiration plus root pressure was measured by differential weighing. The total xylem sap volume, exudated by the decapitated plants, was measured by collecting the exudate without loss. The dry weight of the roots was determined as the base of reference for nicotine and water contents, respectively. For details see ‘Materials and methods’.
and CN⁻-inhibition. Therefore, nicotine had no chance to get in the leaves in the presence of CCCP. The low amount of nicotine, found in CCCP-treated roots, may be due to adsorption of the xenobiotic to the outer root surface and/or to diffusion of a low amount of nicotine into the apparent free space of the root cortex.

Nicotine-induced leaf senescence

It has been shown above that leaves of *E. aureum* accumulate nicotine only up to a certain level (Figs. 3 and 4). This may be regarded as a way to protect the leaves from the toxic effects of nicotine. Yet, sequestration of nicotine in the vacuole of the mesophyll cells (Saunders, 1979) is not such a powerful hindrance against the toxicity of the xenobiotic as it may seem to be in the first place. Rather, nicotine concentrations of the leaves of 3–4 mg g⁻¹ dry weight caused leaf senescence as indicated by several biochemical senescence parameters, which had been determined prior to the beginning of the incubation of the roots in 0.1% nicotine and again after 3 and 5 weeks (Table 3).

The chlorophyll fluorescence ratio $F_v/F_m$ is considered a sensitive indicator of the early stages of leaf senescence (Baker, 1991; Kar et al., 1993). Within 3 weeks, nicotine caused a decrease in the $F_v/F_m$-ratio of the leaves from 0.767 to 0.745 in both plants, while the leaves were still visibly green. During that time span, the total chlorophyll content dropped from 1.40 to 0.81 mg g⁻¹ dry weight, while the chlorophyll $a/b$-ratio increased from 1.9 to 3.2, due to a preferential decline of chlorophyll $b$. Only after 5 weeks, yellowing of the leaves and spot-necrosis were observed. Hence, the ease of *E. aureum* to accumulate nicotine is not fully matched by a likewise outstanding tolerance of the leaves against the toxic, senescence-inducing effects of nicotine accumulation.

Discussion

With 3–4 mg g⁻¹ dry weight (Fig. 4) *E. aureum* beats many *Nicotiana* species with regard to the attainable nicotine concentration in the leaves. In tobacco, the concentration varies between 0.01 and 25.9 mg g⁻¹ dry weight (Jeffrey, 1959; Saitoh et al., 1985) in tobacco species and cultivars. Low concentrations of nicotine have also been found in more than 50 other plant species besides *Nicotiana* (Leete, 1977; Roth et al., 1988; Andersson et al., 2003). In contrast, not even traces of nicotine were found in *E. aureum*. Yet, nicotine is a xenobiotic

### Table 2

Intact *E. aureum* plants treated as described in Table 1, except that the incubation time was 24 h and the plants were subjected to different inhibitor treatments (as listed)

<table>
<thead>
<tr>
<th>Plant number</th>
<th>Nicotin content (mg g⁻¹)</th>
<th>RCFᵃ</th>
<th>Nicotine concentration in the xylem exudate (mg ml⁻¹)</th>
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<tr>
<td></td>
<td>Roots</td>
<td>Leaves</td>
<td></td>
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<tr>
<td>Control: Culture medium (aerated Hoagland solution, pH 7.0)+0.05% nicotine</td>
<td>1 0.91 0.071</td>
<td>1.8</td>
<td>0.403</td>
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<tr>
<td>Anoxic condition: Bubbling N₂ through culture medium (instead of air)</td>
<td>3 1.31 0.033</td>
<td>2.6</td>
<td>0.016</td>
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<tr>
<td>Inhibition of cytochrome oxidase: Addition of KCN (10⁻⁴ M) to the culture medium</td>
<td>5 1.30 0.055</td>
<td>2.6</td>
<td>0.026</td>
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<tr>
<td>Uncoupling of the respiratory chain: Addition of CCCP (10⁻⁵ M) to the culture medium</td>
<td>7 0.37 n.d.ᵇ</td>
<td>0.7</td>
<td>0.023</td>
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</table>

The nicotine contents of the shoots and of the roots was determined after a 24-hours nicotine incubation period. Xylem sap was collected from all plants and its nicotine content was determined. For details see ‘Materials and methods’.

ᵃRoot concentration factor.

ᵇNot detectable.
substance (Sandermann, 1992; Martinoia et al., 2000) with regard to the Golden Potho.

In Nicotiana, the root is the exclusive site of nicotine synthesis and nicotine accumulation in tobacco leaves is preceded by long-distance transport via xylem (Tso and Jeffrey, 1961; Tso, 1972). For nicotine as a xenobiotic, three different modes of transport must principally be considered, namely phloem mobility, xylem mobility and, as a combination of both, amphimobility (Jakob and Neumann, 1987). However, major molecular properties qualify the nicotine molecule as a prime candidate for xylem transport in case of E. aureum, too: (1) its relatively low molecular weight (162 Da), (2) its octanol/water partition ratio ($\log P_{ow}$) of 1.2, (3) its dissociation constant ($pK_a$) of 8.02 (Topp et al., 1986; McFarlene et al., 1987; McCaskill et al., 1988). Assuming a pH of 5.5–6.5 for the apparent free space (apoplast) of the root cortex parenchyma, only between 1% and 3% of the nicotine in the extracellular compartment is present in the lipophilic/uncharged form (=nic). Correspondingly, >95% of total nicotine are nic$^+$, the hydrophilic form (see Eq. 1). Accordingly, within the root cortex only this small proportion of uncharged nicotine is able to penetrate into the symplast (Wink, 1997) as a precondition to pass into the root stele and enter axial long-distance transport in the xylem. An RCF-value of 2–3 (Table 2) supports the interpretation that those nic molecules that overcome the plasmalemma barrier of the root parenchyma cells accumulate in the vacuole (Terry and Robards, 1987) by ion trapping.

The pH gradient between apoplast and vacuolar sap, indispensable for ion trapping, depends on ATP and hence on root respiration to provide it. If the pH gradient is annihilated by the inhibitors, employed here, root pressure dropped to zero in all three cases within the 24 h of nicotine incubation. Consequently, the xylem sap was nicotine-free at the end of the incubation period. In the case of the $N_2$ and CN$^-$ treatments, when the state of full inhibition was reached slowly, the nicotine uptake must have continued for many hours after the onset of the inhibitor treatment and nicotine could accumulate in the leaves. In the case of CCCP, the stage of complete inhibition ATP synthesis, and hence of nicotine uptake, must have been reached much faster because no nicotine at all reached the leaves. An RCF of about 0.7 (Table 2) indicates that a small amount of nicotine must still have passed into the apparent free space of the root cortex, probably adhering there to apolar cell wall structures like lignin and other (perhaps phenolic) constituents of the cell wall (Jakob and Neumann, 1987).

At present, it seems quite unrealistic to assume other hypotheses, such as instance glutationyl-S-transferases or likewise glycosyl- and/or glucuronyl-transferases (Marrs, 1996; Rea et al., 1998; Edwards et al., 2000; Martinoia et al., 2000) for nicotine transmembrane transport in the root cortex since no in vivo nicotine conjugates have been found so far; specific uptake carriers for nicotine have never been invoked. On the other hand, there are convincing experimental evidences for the diffusion of nic into cells driven by the ion-trap mechanism. This has been demonstrated for leaf protoplasts from Catharanthus roseus (Kurkdjian, 1982; McCaskill et al., 1988). Further, calibrated nicotine uptake measurements have been employed for an estimation of intracellular pH-values (Kurkdjian and Guern, 1989; Romani et al., 1998).

ATP is also required at the borderline between xylem parenchyma cells (including the pericycle) and the (apoplastic) xylem elements. Recently, active proton secretion into the xylem has been proposed and it has been suggested that the cations get into the root xylem in a passive way, driven by an electrochemical gradient, which in turn is maintained by ‘anion conductancies’ (Clarkson, 1993; Jahn et al., 1998; Köhler and Raschke, 2000). Anion transport in turn is thought to be

Table 3. The senescence parameters $F_v/F_m$ (maximal quantum yield efficiency), and chlorophyll $a/b$-ratio were determined from mature leaves of the plants which were used to follow-up the time course of nicotine accumulation in E. aureum leaves (Fig. 4)

<table>
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<tr>
<th>Senescence parameter</th>
<th>Time after onset of nicotine application</th>
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<tr>
<td></td>
<td>Zero-time control</td>
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<td></td>
<td>+Nicotine</td>
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<tr>
<td>Chl $a$/Chl $b$-ratio</td>
<td>1.81</td>
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<tr>
<td>Chlorophyll fluorescence ($F_v/F_m$ ratio)</td>
<td>0.7434</td>
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</tbody>
</table>
energized by a Ca\(^{2+}\)-dependent P-ATPase, which secretes H\(^{+}\) into the xylem elements. It is safe to assume that this three-step mechanism then would also be the ‘driving force’ for root pressure and, adopting this model, nic\(^{+}\) would be transferred into the xylem together with the ‘normal’ flux of cations—provided, efflux gates for nic\(^{-}\) are present in xylem parenchyma cells. Recently, non-selective cation channels have been characterized by Davenport and Tester (2000), which possibly could also be claimed for the efflux of nic\(^{-}\) from the root symplast into the vessels.

Although the total water volume, driven through the stem of *E. aureum* plants by transpiration plus root pressure exceeded the water flow, caused by root pressure alone by a factor 4–5 (Table 1), the absolute amount of nicotine translocated in the xylem nevertheless was the same in both cases. This prompted us to conclude that the transpirative water flow is not involved per se in nicotine transport. Since nicotine transport does not at all correlate with the transpiration stream, the possibility can be dismissed that the xenobiotic enters the xylem either by simply leaching through the endodermis of older roots (Pitman, 1982) or by permeation through the water pores of the parenchyma cells of the stele. The latter route can be excluded anyway, since the aquaporins in the plasmalemma (PIPs) discriminate strongly against most organic molecules and ions (Maurel, 1997; Tyerman et al., 1999).

### Acknowledgements

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